Regular Feeding Plays an Important Role in Cholesterol Homeostasis Through the Liver Circadian Clock

Daisuke Yamajuku, Shingo Okubo, Tomonori Haruma, Takahiko Inagaki, Yuji Okuda, Tomoko Kojima, Keiji Noutomi, Seiichi Hashimoto, Hiroaki Oda

Rationale: Peripheral clock control and the relevance of the circadian rhythm to physiology and disease are major questions in mammalian circadian biology.

Objective: We examined the physiological functions of the liver clock.

Methods and Results: We established a suppressed feeding schedule regimen constituting a high-cholesterol diet delivered every 6 hours without changes in energy and cholesterol intake. We found that rats exposed to this regimen developed hypercholesteremia. In the liver, the rhythmicity of expression of several clock genes was disrupted. Furthermore, the nocturnal expression of the CYP7A1 gene, which encodes the rate-limiting enzyme for the conversion of cholesterol to bile acids, was shifted to a diurnal pattern. Indeed, suppression of a regular feeding rhythm increased the secretion rate of very-low-density lipoprotein cholesterol from the liver and decreased the excretion of fecal bile acids.

Conclusions: Our results demonstrated that not only the amount and quality of food but also the timing of meals has crucial health implications. (Circ Res. 2009;105:545-548.)

Key Words: cholesterol homeostasis ■ circadian rhythm ■ lifestyle-related diseases ■ metabolic syndrome

How peripheral clocks impact the incidence of cardiovascular disease and metabolic syndrome remains a fertile area of investigation.¹ Recent studies using genetically modified animals demonstrate that clock genes have a significant role in metabolic regulation.²–⁵ However, the problem is that phenotypes of mice carrying clock gene mutations are not necessarily related to the functions of the respective genes in rhythm generation. Therefore, it is necessary to establish the characteristics of genetically normal animals.

We evaluated the physiological significance of the circadian rhythm by suppressed feeding rhythm without changing the energy intakes. Our experiments demonstrated that regular feeding plays a significant role in cholesterol homeostasis through the liver circadian rhythm.

Methods
Male rats of the Wistar strain (Japan SLC, Japan), 5 weeks of age and weighing ~90 g, were kept on a 12 hour light/12 hour dark cycle (light from 0800 to 2000). Control (C) rats were fed ad libitum, and their food intake was measured over a day. The suppressed (S) group rats were given the same amount of food divided into 4 meals (Zeitgeber time [ZT]0, ZT6, ZT12, and ZT18).

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results
If feeding is a dominant Zeitgeber for the liver clock,⁶ disruption of the regular feeding rhythm should disturb it and have adverse effects on health. We established a feeding regimen involving a high-cholesterol diet (Online Table I) delivered every 6 hour. Cholesterol diet strongly suppresses cholesterol biosynthesis and its circadian oscillation.⁷ We then focused on cholesterol degradation.

This feeding schedule disrupted the regular feeding rhythm without changing the energy intakes (Figure 1A). The body weights of the rats in group C changed rhythmically in a day (Figure 1B). The body weights of the rats in group S increased continuously (Figure 1B). Surprisingly, after 7 days on the experimental diets, the plasma total cholesterol levels were higher in group S than in group C, and this difference persisted (Figure 1C). On day 19, we evaluated metabolic parameters over a 24-hour period. The serum levels of total cholesterol were significantly higher in group S throughout the day (Figure 1D). The ratio of high-density lipoprotein (HDL) cholesterol to total cholesterol was lower (Figure 1D), despite the fact that the absolute level of HDL cholesterol was higher (Online Figure I, A). The levels of serum triglyceride and phospholipids showed circadian rhythmicity in group C;
however, they slightly increased and circadian amplitude was attenuated in group S (Online Figure I, B and C). The serum levels of glucose did not differ between the groups (Online Figure I, D). Agarose gel electrophoresis of serum lipoproteins revealed that very-low-density lipoprotein (VLDL) cholesterol was enriched in group S (Online Figure II).

We used DNA microarray analysis to compare the gene-expression profiles in the livers of groups C and S at ZT14 on day 10. We found that the expression of a key gene in cholesterol degradation, CYP7A1, which encodes the rate-limiting enzyme for the conversion of cholesterol to bile acids,8 approximately 50% decreased in group S livers compared to that in group C (data not shown). We recognized the importance of circadian rhythms in the interpretation of experimental results. Indeed, the CYP7A1 gene displays circadian variation.8 We therefore used the quantitative real-time polymerase chain reaction to profile the circadian expression patterns of CYP7A1 and clock-related genes at different times of day. The nocturnal circadian expression of CYP7A1 shifted to a diurnal peak (Figure 2A). In the livers of group S, the peak D-site binding protein (DBP) mRNA levels decreased and their phase was slightly advanced (Figure 2B). Differentiated embryo chondrocyte (DEC)1 mRNA peaked twice (Figure 2C) and DEC2 mRNA rhythmicity dramatically declined (Figure 2D). Period (PER)1 and PER2 mRNA levels decreased slightly throughout the day (Figure 3A and 3B). Transcriptional factors (such as liver X receptor α, farnesoid X–activated receptor, and small heterodimer partner), which are involved in CYP7A1 gene expression,8 did not change (Online Figure III). Circadian oscillation of CYP7A1 gene expression is mainly regulated by D-site binding protein and DEC2.8,10 Therefore, our results suggested that abnormal rhythmicity of clock-output genes would contribute to the abnormal circadian CYP7A1 expression.

The excretion of fecal bile acids decreased in group S (223.93±11.33 μmol/2 days) compared to that in group C (260.39±12.49 μmol/2 days) (P<0.05). The secretion rates of VLDL cholesterol and VLDL triglyceride from the liver were higher in group S (Online Figure IV). In this experiment, the expression of key genes in cholesterol synthesis (such as sterol regulatory element binding protein 2, 3-hydroxy-3-methylglutaryl-coenzyme A reductase, and low-density lipoprotein receptor)11 did not change between the groups (Online Figure III). This suggests that the hypercholesterolemic effects observed in rats with suppressed feeding rhythms were attributable to the disordered timing of circadian CYP7A1 expression as well as decreased cholesterol degradation and increased cholesterol secretion into blood. With ad libitum feeding, nocturnal eating coincides with the peak of CYP7A1 mRNA, whereas this is not the case with irregular feeding. It has already been known that cholesterol feeding itself does not change in the liver clock.12 Therefore, our results indicated that the feeding schedule itself changed cholesterol metabolism through changes in the liver clock.

We also found that sterol regulatory element binding protein 1 (SREBP1) and fatty acid synthase (FAS) mRNA

### Non-standard Abbreviations and Acronyms

- **DBP** D-site binding protein
- **DEC** differentiated embryo chondrocyte
- **FAS** fatty acid synthase
- **HDL** high-density lipoprotein
- **PER** period
- **VLDL** very-low-density lipoprotein
- **ZT** Zeitgeber time
levels were increased in group S livers (Figure 3C and 3D). PGC1α (peroxisome proliferator-activated receptor γ coactivator 1α) and its target gene involving the β fatty oxidation (such as carnitine palmitoyltransferase 1α [CPT1α]) seems to be slightly advanced in group S livers (Online Figure III). These changes might contribute to the development of hyperlipidemia in group S.

**Discussion**

Clock mutant mice with a C57BL/6J background had a greatly attenuated diurnal feeding rhythm, developed metabolic syndrome and had a significantly increased energy intake.2 Clock mutant mice with an ICR background became less obese on a high-fat diet, because of impaired dietary fat absorption.3 It therefore remains uncertain whether the metabolic abnormality was the consequence of the attenuated diurnal feeding rhythm. Recently, Weitz and colleagues established mice with a liver-specific deletion of Bmal1 and demonstrated that Bmal1 function in the liver is required for circadian regulation of systemic glucose homeostasis.5 However, physiological disorders by abnormal rhythm could be considered to occur because of 2 different causes. The first direct and primary cause is that the clock gene itself induces the pathological effect. The other indirect and systematic cause is that abnormal rhythm itself induces physiological abnormality in genetically normal animals. Therefore, it is
important to establish the physiological significance of the circadian rhythm in genetically normal animals. Schibler and colleagues reported a 4- to 6-hour phase advance in circadian gene expression in the livers of mice under an ultradian feeding rhythm with total food consumption reduced to 74%. However, such experiments are not conclusive in terms of whether the attenuated diurnal feeding rhythm contributes to the metabolic abnormality.

Here, we established a suppressed feeding schedule regimen constituting a high-cholesterol diet delivered every 6 hours without changes in energy intake. We tried to find the effect of nonphysiological feeding pattern on lipid profile and to make some insights into the mechanism by measuring circadian rhythm of various substances especially in the liver. Consequently, this hypercholesterolemia would be attributable to the disordered timing of circadian CYP7A1 expression, as well as decreased the excretion of fecal bile acids. Our chrononutritional approach, in a rat model, showed that an irregular eating style disturbed the liver clock and increased the risk of arteriosclerosis. Moreover, our results indicated that not only the amount and quality of food but also the timing of meals has crucial health implications.

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Disclosures
None.

References
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EXPANDED MATERIALS AND METHODS

Experimental design of suppression of regular feeding rhythm

Male rats of the Wistar strain (Japan SLC, Japan), 5 weeks of age and weighing about 90 g, were kept on a 12 h light 12 h dark cycle (light from 0800–2000). They were initially fed a basal diet for 3 days ad libitum. Free access to water was provided throughout the experiment. Before being divided into 2 groups matched for plasma cholesterol concentrations and body weight, they were fed high cholesterol diets for 11 days ad libitum. The compositions of the diets are shown in Online Table I. All animals were fed a high cholesterol diet. Control (C) rats were fed ad libitum, and their food intake was measured over a day. The suppressed (S) group rats were given the same amount of food divided into 4 meals (ZT0, ZT6, ZT12, and ZT18). Food intake was measured at 6 h intervals (ZT0, ZT6, ZT12, and ZT18). Body weight was measured at 12-h intervals (ZT0 and ZT12). Blood samples were collected from the tail vein at ZT6 after 4-h fasting. The rats were killed by decapitation at 4-h intervals after 4-h fasting on days 19–20. Their livers were immediately removed, frozen in liquid nitrogen, and stored at –80°C until assayed, and blood was collected from the cervical wound. Serum was prepared by centrifugation at 1500 × g for 10 min.

RNA analysis

Total RNA was extracted following the method of Chomczynski and Sacchi 1. The mRNA levels were also analyzed by quantitative real-time polymerase chain reaction as described previously 2. The β-ACTIN and GAPDH mRNA levels were not affected in the present investigation; therefore, we used them as the normalization standard. The sequences of the primer sets used are shown in Online Table II.

Biochemical analysis

The serum total cholesterol, HDL-cholesterol, phospholipid, triglyceride, and glucose were enzymatically determined by using a commercial kit (Cholesterol C-test, HDL–cholesterol C-test,
phospholipid C-test, Triglyceride E-test, and Glucose CII-test; Wako Pure Chemical Industries, Osaka, Japan). Fecal bile acids were determined enzymatically by the method of Sheltawy and Losowsky \(^3\).

**Agarose gel electrophoresis of serum lipoproteins**

Agarose gel electrophoresis was carried out by using Universal film from Helena Laboratories (Saitama, Japan). After the agarose gel was run, lipoprotein-cholesterol was stained with Co-Cholest-A (Nippon Chemiphar, Tokyo, Japan).

**Secretion rate of very low density lipoproteins (VLDL)**

Tyloxapol, which is the same polyethylene glycol as Triton WR-1339, was injected into the external jugular vein of rats at a dose of 400 mg/kg body weight in a 200 g/L solution of 0.15 mol/L NaCl at ZT6 after a 4-h fasting period \(^4\). The secretion rate of VLDL lipid was calculated using the following equation: the secretion rate of VLDL (mg/h) = \((C_6V - C_0V)/6\), where \(C_6\) and \(C_0\) are milligrams of lipid per milliliter of plasma at 6 h after tyloxapol treatment and just before tyloxapol treatment, respectively; \(V\) is the plasma volume (in milliliters calculated at 4 mL per 100 g of body weight).

**Statistics**

The results for each parameter are expressed as the mean ± SEM. Student’s \(t\) test was performed to compare groups C and S. Statistical significance is displayed as \(P < 0.05\) (1 asterisk), \(P < 0.01\) (2 asterisks), and \(P < 0.001\) (3 asterisks). Rhythmicity was tested by a one-way ANOVA.
References


ONLINE FIGURE LEGENDS

**Online Figure I.**
Circadian metabolic parameters in rats suppressed a regular feeding regimen. On day 19, the circadian profiles of serum HDL cholesterol (A), triglyceride (B), phospholipids (C), and glucose (D) are shown. The values for groups C (open circles) and S (filled circles) are mean ± SEM of 4 rats, respectively.

**Online Figure II.**
Agarose gel electrophoresis of serum lipoproteins from groups C and S at ZT14 and ZT 2'. Lipoprotein-cholesterol was stained enzymatically by using Co-Cholest-A. α; HDL; pre β; VLDL.

**Online Figure III.**
Hepatic circadian gene expression profiles in rats suppressed a regular feeding regimen. On day 19, the livers of the rats in groups C and S were collected at 4 h intervals. Real-time PCR was used to determine mRNA levels of LXRα, FXR, SHP, SREBP2, HMGCoA, LDLR, PGC1α, and CPT1α. The open and solid horizontal bars indicate the light and dark periods, respectively. The values for group C (open circles) and S (filled circles) are displayed as relative abundance (mean ± SEM of 4 rats).

**Online Figure IV.**
The suppression of a regular feeding rhythm increases the secretion rate of VLDL cholesterol from the liver. On day 17, tyloxapol was injected at a dose of 400 mg/kg of body weight. Accumulation of plasma cholesterol (A) and triglyceride (B) after the injection of tyloxapol. The secretion rate of VLDL-cholesterol (C) and VLDL-triglyceride (D) was determined. The values for group C (open circles) and S (filled circles) are the mean ± SEM of 7 and 8 rats, respectively.
Online Figure II

The figure shows gel electrophoresis results for different groups labeled as ZT14 group C, ZT14 group S, ZT2' group C, and ZT2' group S. Arrows indicate the locations of α, preβ, and origin bands.
Online Figure IV

A

Plasma cholesterol (mg/dL)

Time after injection of Tyloxapol (h)

B

Plasma triglyceride (mg/dL)

Time after injection of Tyloxapol (h)

C

Secretion rate of VLDL cholesterol (mg/100g BW x h)

group C

group S

D

Secretion rate of VLDL triglyceride (mg/100g BW x h)

group C

group S
<table>
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<th>High cholesterol</th>
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<td>(g/100g diet)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
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<tr>
<td>Vitamin mixture 1)</td>
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<td>1</td>
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<td>Mineral mixture 2)</td>
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<td>5</td>
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<td>Cellulose</td>
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<td>Starch</td>
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<tr>
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<tr>
<td>Sodium cholate</td>
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1) Vitamin mixture: AIN-93TM
2) Mineral mixture: AIN-93TM
| **Online Table II. Primer sequences for quantitative PCR** |
|---------------------------------|---------------------------------|
| **Forward primer** | **Reverse primer** |
| CYP7A1 | TGTGTGAGGGACCAGGTCTCT | AGCTCCAAAAGGTTGCAGGA |
| DBP | CTCTAGGGACACACCCAGTCTCT | AGGCTTCAATTCTCCTCTGAGA |
| PER1 | ACCAGCTCAAGGCTTAGGAGCT | TGGGATTTGGAGAGACCACCTTC |
| PER2 | CAACCTTTGTCTGTCATATGAGG | CGTTAGAAACACAGCTCTCCAC |
| DEC1 | GGGAAAAACTGTGTGCCAGTC | GCCGATCGGCTGGAAGTCCAC |
| DEC2 | AACCCCTTTGTCCCATGTCTC | GCCCATGGAAGCCATCTCCAC |
| LXRα | TCCGAGATCTGGGGATGCTCA | TGGGATTTGGAGAGACCACCTTC |
| FXR | AGCCACAGATCTCCCTGCTCG | ACAGGCATCTCGGATACCTCA |
| SHP | CAGCTTTGATTTCCCTGCTTTC | GTCTGGAGGAATTTCCTGCC |
| SREBP2 | CTGCCAACCTACAGACCTGCTT | GCCGTGGAAGACCTTTTGGAG |
| HMGCoAR | TGCACAGACTTCTCAGACGTG | TTCGTCGAAAACACAGCTTCC |
| LDLR | GGTCAGCCTGGAGATGATGT | GCTTTGAGCAACAGAGACCA |
| SREBP1 | GGAGCCATGGGATGGACTTGGGATGT | AGGAGGCTTTCCAGAGAG |
| FAS | TTCTCAGGGCCGTGATAAATGC | GTAGGCGGTTGCTAGACAAC |
| PGC1α | CATTTTGCAACAGCAAAAGCCA | GCGGTGGTGTATGGGACTTCTT |
| CPT1α | CTGTGAAAGCCTTTGGTGGAT | GGAAGCTGGCAGGCAAATGGA |
| GAPDH | GATACTGAGAGCAAGAGAG GCC | GATGGTATTCCAGAGAGAGGG |
| β-ACTIN | GGTCGTACCAGCTGGCATTT | GCTGGTGCCGATCTCTGAG |